Presence of Antioxidant Materials in Bacteria

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ABSTRACT

Fourteen species of gram-positive and gram-negative bacteria from nine genera were investigated to determine if they produced compounds that have antioxidant activity. Washed bacterial cells were extracted with methanol for 24 hr. This extract was evaporated to dryness and extracted with benzene. The fractions were added to lard and incubated at 58 C. Antioxidant activity was determined by prolongation of the period required for the initiation of rancidity as measured by changes in peroxide value. The methanol soluble-benzene soluble fractions of *Bacillus cereus, Lactobacillus dextranicum, Micrococcus freudenreichii* and *Sarcina lutea* showed considerable antioxidant activity. Methanol fractions of three *Pseudomonas* species showed considerable activity that could not be extracted with benzene. The possibilities of using microbial growth on fats as well as extracts of microorganisms added to fats as antioxidants are discussed.

INTRODUCTION

The number of effective antioxidants that can be added to food products is limited and new compounds would be very useful. Lea (1), in 1938, suggested that because of the reducing action of microorganisms, the growth of a microbial flora on a fatty medium could lead to an inhibition of the oxidation of the fat by preventing the formation of peroxides and partially or completely destroying preformed peroxides.

In spite of this long-standing suggestion, few papers have appeared in which the antioxidant activity of microorganisms has been investigated. An oil soluble fraction of *Mycobacterium phlei* added to cottonseed oil protected it against oxidation (2) with the antioxidant activity apparently associated with the lipid component of the cell. Tatarenko and coworkers (3) were able to prevent rancidity in lard and butterfat by adding the nonsaponifiable portion of ether-extracted mycelia of *Naumoviella oleaginosa* to them. The antioxidant, 2-(hydroxy-2-methoxy-3,4-methylenedioxyphenyl)-benzofuran, isolated from baker's and brewer's yeast, was effective in protecting fats or food containing fats (4,5), and an oil soluble extract of *Aspergillus oryzae* contained a factor that prevented oxidative rancidity (6).

Recent investigations from our laboratory have shown that a large number of microorganisms have the ability to decompose the peroxides present in fresh and rancid lard (7,8). This peroxide-decomposing activity of microorganisms appears to be enzymatic in nature (9).

In attempting to extract the peroxide-decomposing material, it became apparent that a distinct antioxidant material also was present. The present investigation was undertaken to examine a wide range of bacteria as potential sources of nonenzymatic antioxidant materials. The preparation of these materials minimized the possibility that the activity was enzymatic. Crude extracts were prepared for comparison of the relative efficiency of the material from each source.

MATERIALS AND METHODS

Media for Growth

The microorganisms studied were representative strains from our culture collection and are listed in Table I. The media for production of cells were: Medium 1, Difco tryptose phosphate broth. Medium 2, Difco veal infusion broth. Medium 3, BBL APT broth. Medium 4, Case peptone, 1.0 g; 1 M phosphate buffer (pH 7, equimolar Na:K), 5 ml; distilled water, 94 ml. All media were sterilized by autoclaving at 121 C for 15 min.

Purification of Methanol and Benzene

Although reagent grade chemicals meeting American Chemical Society's requirements were used, the benzene and methanol contained substances that accelerated the oxidation of lard. To remove most of these prooxidants, benzene was passed through a celite-sulfuric acid column (10) and then distilled. Methanol was treated by the method of Lund and Bjerrum (11). The residue from 100 ml of benzene, or 400 ml of methanol, oxidized 15 g of lard to a peroxide value (PV) of 15 in 5.3 days and 2.3 days, respectively, as compared to

TABLE I

Bacterial Species Employed and Methods of Producing Cells for Isolation of Antioxidant Activity

Bacteria	Medium used[a]	Incubation conditions
Staphylococcus aureus No. 63 and No. 66	1	35 C; 24 hr, shaken[b]
Sarcina lutea No. 112	1	25 C; 24 hr, shaken[b]
Micrococcus cryophilus No. 90	1	20 C; 24 hr, shaken[b]
Micrococcus freudenreichii No. 115	1	20 C; 24 hr, static[c]
Bacillus cereus No. 283 and No. 284	2	25 C; 24 hr, shaken[b]
Escherichia coli No. 107	2	25 C; 24 hr, shaken[b]
Serratia marcescens No. 279	2	25 C; 24 hr, shaken[b]
Pediococcus cerevisiae No. 270	3	25 C; 48 hr, static[d]
Leuconostoc dextranicum No. 268	3	25 C; 48 hr, static[d]
Pseudomonas ovalis No. 36	4	20 C; 24 hr, shaken[b]
Pseudomonas fragi No. 43	4	20 C; 24 hr, shaken[b]
Pseudomonas species No. 92	4	20 C; 24 hr, shaken[b]

[a]The composition of each medium is given in the text.

[b]Two hundred milliliters of medium were dispensed into 1-liter Erlenmeyer flasks; after inlculation, the flasks were incubated on a rotary shaker at 200 rpm.

[c]One liter of medium was dispensed into 3 liter low form culture flasks.

[d]Two liters of medium were dispensed into 3 liter low form culture flasks.

10 days for the control. The purified solvents required 8.8 days and 6.7 days, respectively.

Preparation of Antioxidant Fractions and Assay of Antioxidant Activity

Appropriate media were inoculated with 18-24 hr cultures of the bacteria and incubated as indicated in Table I. Several solvents, including hexane, petroleum ether, methanol, acetone and benzene were examined for their ability to extract an antioxidant material. Sonication of the cells before extraction was of no value and the following procedure gave the best results. The cells were harvested by centrifugation and washed twice with 0.005 M phosphate buffer (pH 7, equimolar Na/K), and the cell pellets kept frozen until needed. Approximately 250 ml of methanol were added to 10-50 g wet weight of thawed cells and agitated on a magnetic stirrer for 24 hr at room temperature. The cellular debris was removed by centrifugation and the methanol removed by evaporation from a rotary evaporator at 50-60 C. Forty milliliters of benzene were added to the residue, mixed, and the material soluble in benzene removed. Both fractions were evaporated to dryness by rotary evaporation. Thus, a methanol soluble-benzene soluble fraction and a methanol soluble-benzene insoluble fraction were obtained. Fifteen grams of melted lard were added to a known weight of each fraction, the flasks rotated 5 min at 50-60 C, and the resulting mixtures placed in covered jars and incubated at 58 C in the dark. At intervals, approximately 200-250 mg of fat were removed and the PV determined by the iodometric method of Lea (12).

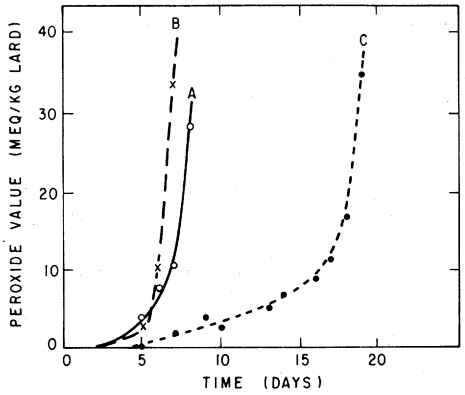


FIG. 1. Antioxidant activity of fractions from *Staphylococcus aureus* No. 63. A, methanol soluble-benzene insoluble, 253 mg; B, control; C, methanol soluble-benzene soluble, 275 mg.

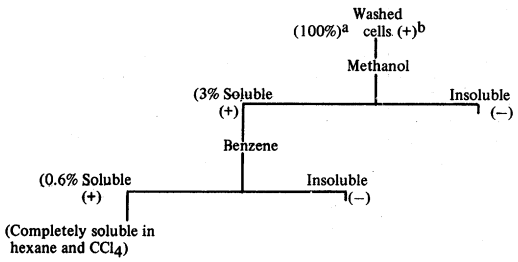
Mathematical Treatment of Data

The line of best fit was determined by the least squares analysis of regression (13).

RESULTS

The extractibility of antioxidant material from *Bacillus cereus* by different solvents is summarized in Scheme 1.

Initial extraction of cells with benzene yielded very little activity. When benzene was used to extract the methanol-soluble material from the *Pseudomonas* species, the extraction was incomplete and variable results were obtained.



[a]Approximate per cent by weight of starting material.
[b]Antioxidant activity present.

Scheme I

Therefore, only the methanol fraction of these three cultures was tested in subsequent experiments. When cells were ashed, the antioxidant activity was destroyed. The supernatant fluid of the culture was examined for antioxidant activity, but none was detectable, even when an entire lot of growth medium was evaporated to dryness, extracted and tested.

In a typical pattern of fat oxidation there is an induction period, during which little or no change in peroxide value (PV) occurs. This is followed by a shorter period in which the rate of change in PV rapidly accelerates, reaching a high PV a short time after the oxidation begins. In Figure 1, a typical experiment with the methanol soluble-benzene soluble and methanol soluble-benzene insoluble fractions of *Staphylococcus aureus* No. 63 shows how these fractions affect the induction period. The primary effect was typical of known antioxidants in that it extended the induction period rather than decreasing the rate of oxidation once it had begun. All of the other microorganisms tested had a pattern similar to that of *S. aureus* although they varied in the time at which the upswing of the curve occurred.

There is no specific PV at which a fat can be considered rancid. However, by the time the lard in these experiments had reached a PV of 15, a rancid odor was detectable and the PV was increasing rapidly. Since different batches of fat will not oxidize at the same rate, the data from each experiment were normalized by arbitrarily adjusting the control to a PV of 15 in 10 days and then adjusting the other results in the same ratio. Utilizing this procedure, the effect of increasing concentrations of the methanol soluble-benzene soluble fraction of several microorganisms on the onset of rancidity was determined. Figure 2 indicates the linear responses obtained with three of the cultures. A linear response similar to that of *B. cereus* No. 284 was given by *B. cereus* No. 283, *Lactobacillus dextranicum, Micrococcus freudenreichii, Sarcina lutea* and *Escherichia coli*, and the methanol extract of *Pseudomonas fragi*. The two *S. aureus* strains were similar to *Micrococcus cryophilus*, and *Pediococcus cerevisiae* was similar to *Serratia marcescens*. The methanol extract of *Pseudomonas ovalis* and *Pseudomonas* sp. No. 92 gave a consistent curvilinear response as is shown in Figure 3.

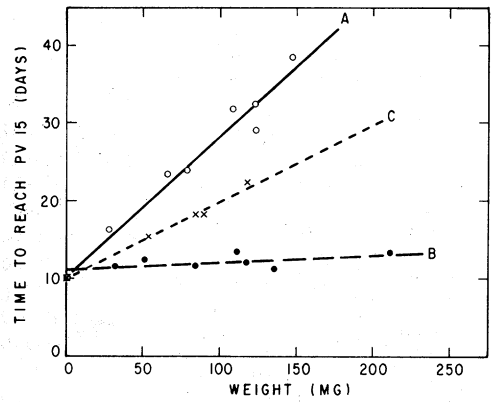


FIG. 2. Effect of varying concentrations of the methanol soluble-benzene soluble fraction from different bacteria on the stability of lard. A, *B. cereus* No. 284; B, *S. marcescens*; C, *M. cryophilus*.

The activity of the fractions from the various microorganisms is compared at the 100 mg level in Table II. With the exception of *E. coli*, the benzene insoluble fractions had little or no activity. Fractions from *M. freudenreichii, S. lutea, E. coli, B. cereus* No. 283 and No. 284, *L. dextranicum, P. fragi, P. ovalis* and *Pseudomonas* sp. No. 92, which protected lard against oxidation for more than 20 days, have strong antioxidant activity and warrant further investigation.

Lard reached a PV of 15 in 43.0, 33.5 and 22.2 days when butylated hydroxyanisole

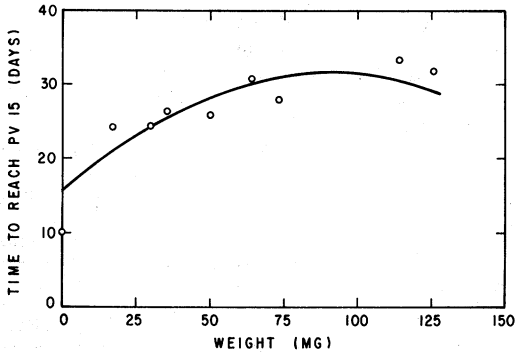


FIG. 3. Effect of varying concentrations of the methanol soluble material from *P. ovalis* on the stability of lard.

TABLE II

Comparison of the Ability of Fractions[a] From Microorganisms to Stabilize Lard

Microorganisms	Number of days for oxidation of lard[b]		
	Benzene insoluble	Benzene soluble	Methanol soluble
Micrococcus freudenreichii No. 115	10.8	23.4	---
Sarcina lutea No. 112	9.9	21.4	---
Micrococcus cryophilus No. 90	10.3	19.9	---
Staphylococcus aureus No. 66	10.5	18.6	---
Staphylococcus aureus No. 63	9.4	15.6	---
Escherichia coli No. 107	16.7	22.9	---
Serratia marcescens No. 279	10.8	12.0	---
Bacillus cereus No. 284	10.8	28.4	---
Bacillus cereus No. 283	10.7	26.4	---
Leuconostoc dextranicum No. 268	11.2	24.4	---
Pediococcus cerevisiae No. 270	11.1	12.8	---
Pseudomonas sp. No. 92	---	---	34.0
Pseudomonas ovalis No. 36	---	---	31.2
Pseudomonas fragi No. 43	---	---	29.7

[a]100 mg/15 g lard.

[b]Time for PV of lard to reach 15. Data are normalized to a value of 10 days for the controls.

(BHA), butylated hydroxytoluene (BHT) and α-tocopherol, respectively, were added at a concentration of 0.005 wt. %; the control value with no antioxidant was 10.0 days.

DISCUSSION

Although there are definite differences in concentration of antioxidant materials found in the crude extracts from the various bacteria, it remains to be determined whether these differences were a function of concentration, ease of extraction, presence of prooxidants which were simultaneously extracted, or to actual chemical differences in the compounds involved. On the basis of the results reported here, the extracts from *P. ovalis* were the most active since as little as 25 mg/15 g of lard extended the shelf life beyond 20 days. At higher concentrations, however, the curvilinear response of this extract became a factor and at 100 mg/15 g *Pseudomonas* sp. No. 92 gave better protection. The lack of any antioxidant activity in the supernatant fluid indicates that the material is intracellular and the cells must be harvested and extracted to obtain it. Since the active material is destroyed by ashing, it is not a simple inorganic substance; however, the harsh solvent extraction makes it unlikely that it is enzymatic.

The difficulties in extraction of antioxidant materials were greatest with the four gram-negative bacteria (*E. coli* and the three *Pseudomonas* species). Whether or not this problem was related to the known higher lipid content of the cell walls of gram-negative bacteria (14) awaits further study.

These data indicate that retardation of oxidation by microorganisms actively growing on the fatty medium, as suggested by Lea (1), would require extensive growth of the culture to have an appreciable effect. Such growth would likely be undesirable because of other changes such as lipolysis or proteolysis that accompany metabolism. However, on the surface of some meats, such as aged hams (15), dry sausages (16), and other foods normally accompanied by extensive microbial growth, a significant amount of antioxidant activity might develop.

On a weight basis, the bacterial fractions were not as active in protecting fats against oxidation as were BHT, BHA, and α-tocopherol. However, the microbial fractions were crude preparations and must be further purified before a suitable comparison can be made.

The work reported here establishes the potential of bacteria as sources of antioxidant materials. The chemical nature of the compound or compounds responsible is being actively investigated, and from these studies more definite information on the economic aspects should be forthcoming.

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